DNA Diagnostics to Identify Internal Feeders (Lepidoptera: Tortricidae) of Pome Fruits of Quarantine Importance

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ABSTRACT A diagnostic polymerase chain reaction (PCR) method is presented for differentiating among the North American internal apple-feeding pests codling moth, *Cydia pomonella* (L.); oriental fruit moth, *Grapholita molesta* (Busck); lesser appleworm, *Grapholita prunivora* (Walsh); and cherry fruitworm, *Grapholita packardi* Zeller. An ≈470-bp fragment of mitochondrial cytochrome oxidase subunit I (COI) was sequenced in three to six specimens of each species. Consistent and diagnostic differences were observed among the species in two regions of COI from which forward and reverse primers were designed to amplify a 112–116-bp segment of the gene. The primer sets were used to selectively amplify DNA from specimens of diverse geographic origin for each corresponding target species. Protocols were adapted for conventional and quantitative PCR, the latter being substantially faster. The method was validated as a decision-making tool for quarantine identifications for Mexico by representatives of their phytosanitary agency (Sanidad Vegetal). The method can facilitate identification of intercepted internal feeding Lepidoptera in apple and pear for many other importing nations.

KEY WORDS quantitative PCR, quarantine pests, mitochondrial COI, pome fruit

IN NORTH AMERICA, APPLES AND pears are attacked by four species of tortricid internal fruit feeders: codling moth, *Cydia pomonella* (L.); oriental fruit moth, *Grapholita molesta* (Busck); lesser appleworm, *Grapholita prunivora* (Walsh); and cherry fruitworm, *Grapholita packardi* Zeller (Weires and Riedl 1991, Beers et al. 1993). The first two species are Palearctic and the latter two Nearctic in origin, but interception of any of the four within pome fruits may be a quarantine issue when exporting these fruits from the United States (Smith et al. 1992, Santos et al. 1998, NHC 2004) or to different regions within the United States (Krawczyk and Johnson 1996) (Table 1).

Accurate identification of pests is essential before quarantine restrictions can be applied to a fruit shipment, especially if the unknown has characteristics of a quarantine pest. Most procedures rely on morphological characterization of adults or late instars for species identification. This is particularly true with the four species addressed here because the smaller larval stages (L_1–L_3) often cannot be reliably separated to species with currently used morphological traits (S. Passoa, personal communication). Unfortunately, often the interceptions of internally feeding Lepidoptera in pome fruits at border inspection stations of Mexico are small larvae and are difficult to identify (E. Vega, personal communication). Occurrence as small larvae at or near harvest of pome fruits is possible for all four species because the three *Grapholita* spp. tend to fly in mid- to late summer (Chapman and Lienk 1971), codling moth often has a partial third generation in Washington and Oregon (Beers et al. 1993) and a partial fourth generation in warmer parts of California (University of California 1991). Hence, there is a need to supplement insect identification procedures in support of quarantine decisions with a means of rapidly identifying immature life stage infestations to the species level. This capability is important to both the importing and exporting countries; decisions that protect a country from an exotic pest also determine the fate of an infested consignment. Incomplete identifications result in necessarily cautious decisions by the importing nation, and this may result in many thousands of dollars worth of fruit needlessly destroyed or turned away. A rapid technique is required that is diagnostic to species and uses minimal quantities of potentially poor-quality material from any life stage.

Over the past decade, the use of molecular-based approaches to identify unwanted pests has increasingly been used on both incoming and outgoing produce. The rapid nature of the methods, the ability to identify any life stage of the organisms, and the uniformity of techniques are keys to their increasing use (Armstrong et al. 1997, Gleeson et al. 2000). Nuclear

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Table 1. Example of countries where North American pome and stone fruits have quarantine restrictions due to internal fruit feeding Tortricidae

Pest	North America and Caribbean	South America	Eurasia	Other
C. pomonella	Cuba	Ecuador	China	Egypt
	Dominican Republic	Brazil	Taiwan	
		Colombia	Korea	
		Venezuela	Japan	
G. molesta	Canada	Peru	European Union	S. Africa
	México		Russia	
	Cuba		Indonesia	
G. prunivora		Peru	European Union	New Zealand
		Brazil	Japan	Australia
		Argentina	Indonesia	
G. packardi		Argentina	European Union	New Zealand
			Indonesia	Australia

Data are from NHC (2004).

ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) have been widely used for taxonomic and phylogenetic studies in insects (Hwang and Kim 1999), including tortricids (Gleeson et al. 2000; Kruse and Sperling 2001, 2002), and consequently there is an increasing number of DNA sequences from these regions available. These sequences are useful for developing polymerase chain reaction (PCR) primers for both phylogenetic and practical reasons: DNA sequences are likely to be similar among closely related taxa than distantly related taxa, and multiple copy genes (rDNA) or genes from the mitochondria that occur in multiple copies within each cell are often easier to amplify by PCR, especially with limited amounts of DNA or degraded DNA (Unruh and Woolley 1999). For the four target species, mtDNA sequences in GenBank (Benson et al. 2004) included 22 haplotypes of the noncoding AT-rich region for codling moth, 11 haplotypes of cytochrome oxidase subunit I (COI) for codling moth, and one haplotype of COI for oriental fruit moth. In addition, 14 COI haplotypes from 12 species of leafrollers known to attack pome fruits around the world were available for comparison and aid in primer design.

Mitochondrial COI is by far the most commonly used sequence for quarantine applications. Examples include *Tetranychus* mites (Lee and Lee 1997), *Bactrocera* fruit flies (JeomHee et al. 2000), *Liriomyza* leafminers (Scheffer et al. 2001), and tortricid leafrollers (Sperling and Hickey 1995). Other mitochondrial genes also have proven to be useful, such as NADH in *Rhagoletis* fruit flies (Salazar et al. 2002). Most diagnostic protocols for arthropods have relied on restriction analysis of a PCR product (PCR-restriction fragment-length polymorphism) amplified using universal primers (Simon et al. 1994). However, some recent studies have used PCR with species-specific primers, which overcome the need of postamplification digestion and reduce the time required for diagnosis (Fettene and Temu 2003, Kampen et al. 2003). The introduction of quantitative PCR (or real-time PCR; qPCR) along with the design of sequence-specific primers and probes has further increased speed and accuracy of phytosanitary diagnoses, although this has been done mainly with plant pathogens (Schaad et al. 2003, Mavrodieva et al. 2004).

A rapid and reliable method based on PCR with species-specific primers is presented to discriminate among the four tortricid internal fruit feeders of apple and pear that occur in North America. The methodology was developed for conventional PCR as well as for qPCR, the latter providing identification in 4 h from the time the intercepted unknown specimen is received in the laboratory.

Materials and Methods

Insects. Specimens were obtained as larvae from laboratory colonies (codling moth, lesser appleworm, and oriental fruit moth) and field collections (cherry fruitworm) and as adults from museum collections, reared from fruit, and taken from sticky traps (all species; Table 2).

DNA Extractions. Total DNA was obtained from single specimens following protocol A for isolation of genomic DNA from insects of the DNeasy tissue kit (QIAGEN, Valencia, CA). Fresh, frozen, or ethanol-preserved larvae from laboratory colonies or field populations; legs from pinned adult museum specimens; and adults from sticky traps were used. In addition, a rapid (<3-h) and inexpensive (<$0.50) DNA extraction procedure for 70% ethanol-preserved larvae (condition of specimens sent for diagnosis) was adapted from Kazmer et al. (1995) by using Chelex 100 Resin (Bio-Rad, Hercules, CA). Briefly, the 3–5 mm larva was ruptured with a dissecting needle (or a 2-mm segment was cut from the midsection) and rinsed in distilled H_2O and blotted on sterile filter paper three times. The specimen was placed in a sterile 0.5 ml Eppendorf tube and macerated with a pestle after adding 100–200 μl of 5% Chelex and 3–6 μl of Proteinase K solution RNA grade (20 mg/ml). The sample was vortexed for 3 s and then the resin was spun to the bottom of the tube. The sample was incubated for 30 min (fresh, frozen, or ethanol preserved) to overnight (museum and sticky trap specimens) at 55°C, boiled for 10 min, resin spun down again, and kept at −20°C until used.

Table 2. Source locality of specimens used for sequencing and primer validation

Species	Collection site	Country	*n*	Life stage	GenBank accession no.
C. pomonella					
	Yakima, WA[a,c,d]	United States	16	L	AY728144
	Sebastapol, CA	United States	1	A	
	Wooster, OH	United States	1	A	
	Layrac	France	1	A	
	Oxsai[a]	Kazakstan	1	A	AY728145
	Ketman	Kazakstan	1	A	
	Targabatai	Kazakstan	1	A	
	Guzeripl, Caucasus	Russia SSR	1	A	
	Mezmaj, Caucasus	Russia SSR	1	A	
	Anapa	Russia SSR	1	A	
G. molesta					
	Fresno, CA[a,c,d]	United States	20	L	AY728146
	Bridgeton, NJ[c]	United States	1	L, A	
	East Lansing, MI	United States	1	L	
	Saint Privat, Ardiche[a]	France	1	A	AY728147
G. prunivora					
	Wapato, WA[a,d]	United States	12	A, L	AY728148
	Tacoma, WA	United States	1	A	AY728149
	Seattle, WA	United States	1	A	AY728150
	Coles, Co., IL[b]	United States	1	A	AY728151
	Biglerville, PA	United States	1	A	AY728152
	Québec, Canada[b]	Canada	1	A	AY728153
G. packardi					
	Seattle, WA	United States	2	A	AY728154
	Lace Co., MI	United States	5	L, A	AY728155–6
	Coles Co., IL[b]	United States	1	A	
	Houston, TX[c]	United States	1	A	

n is number of specimens used for primer validation per source area. Also shown are the life stages used (A, adult; L, larva) and sequences submitted to GenBank.
[a] Voucher specimens submitted to M. T. James Entomological Collection (Washington State University, Pullman, WA).
[b] Pinned specimens less leg used remain in collection of University of Minnesota (W. E. Miller).
[c] Additional specimens from these localities in USDA–ARS Systematic Entomology Collection.
[d] Laboratory colonies kept at USDA–ARS (Wapato, WA).

PCR Amplification of COI. The universal primers C1-J-1718, 5′-GGAGGATTTGGAAATTGATTAGTTCC-3′; and C1-N-2191, 5′-CCCGGTAAAATTAAAATATAAACTTC-3′ (Simon et al. 1994) were used to amplify ≈525 bp of the COI gene. qPCR amplification and melting curve analysis (see below) used the SmartCycler (Cepheid, Sunnyvale, CA) and were analyzed with SmartCycler Software (version 1.2f). Amplifications were performed in 25-μl reaction volumes containing 10 mM Tris-HCl, 50 mM KCl, 3.5 mM $MgCl_2$, 0.001% gelatin, 0.25 μl of 1:400 SYBR Green I, 200 μM of dNTPs (Boehringer Mannheim, Ridgefield, CT), 0.625 U of Platinum *Taq*DNA polymerase (Invitrogen, Carlsbad, CA), 0.2 μM each primer, and 4 μl of template DNA. The PCR temperature program was 95°C for 120 s and 40 cycles of 95°C for 15 s, 50°C for 30 s, and 72°C for 45 s. PCR was followed by melt-curve analysis (see below), which consisted of increasing the temperature of the sample from 60 to 95°C in steps of 0.2°C s^{-1}. The resultant PCR product was used for DNA sequencing.

Total fluorescence produced by the intercalation of SYBR Green into double-stranded DNA was monitored and provided a measure of PCR product (=amplicon) formation. The cycle-threshold, C_T (the PCR cycle number at which the level of fluorescence of the sample was 30 fluorescence units above background, as recommended by manufacturer), was used to denote when target or nontarget DNA was detected. C_T also reflects PCR conditions and DNA quality and concentration, and this fact underpins the quantitative nature of qPCR (C_T will be reached sooner with higher target DNA concentrations).

Melting curve analysis provided a second criterion to determine presence or absence of a specific target sequence and as a measure of the quality of the PCR. In general, each amplicon exhibits a characteristic T_m, the temperature of 50% melting, which is dependent on GC content, length, and sequence of the product (Li et al. 2003, Mavrodieva et al. 2004). The T_m of a given PCR product also depends on the experimental conditions (Li et al. 2003); hence, it must be determined for each experimental protocol. T_m and melting curves were compared with two or more electrophoretic separations for each target amplicon to ensure that the melting peak(s) observed corresponds to a single band of expected size

DNA Sequencing. Double-stranded PCR products were purified (QIAquick PCR purification kit, QIAGEN) and sequenced (ABI PRISM BigDye Terminator v3.1 cycle sequencing kit) in an ABI PRISM 310 Genetic analyzer (Applied Biosystems, Foster City, CA) following manufacturers' protocols. All fragments were sequenced in both directions. Two codling moth, two oriental fruit moth, six lesser appleworm, and three cherry fruitworm specimens were sequenced.

Alignment and Primer Design. Sequences were aligned manually and numbered according to the mitochondrial COI sequence of *Drosophila yacuba* Burla (Clary and Wolstenholme 1985). In addition, 11 GenBank sequences of COI from codling moth (AF4978436–46) and one from oriental fruit moth (AF499023) were considered during primer design. Diagnostic primers were designed using PRIMER3 (Rozen and Skaletsky 2000). BLAST (Altschul et al. 1990) searches against diagnostic primer sequences were done in GenBank. Primers were synthesized by Sigma-Genosys (Woodlands, TX).

PCR Amplification with Diagnostic Primers. The qPCR protocol was developed using the Cepheid RapidCycler and also was validated in an ABI7000 quantitative PCR instrument (Applied Biosystems). DNA of each of the four species was tested with the four sets of diagnostics primers. Amplifications using the diagnostic primers were performed and analyzed as described above for universal primers, except the PCR program: 95°C for 120 s and 30 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s (extension time of 30 s was used for the ABI7000). Again, amplification product specificity was validated by melt curve analysis and agarose electrophoresis as described below.

Because qPCR capabilities are not present in all diagnostic laboratories, protocols also were developed for conventional PCR. In this case, amplifications were performed in 25-μl reactions with 10 mM Tris-HCl, 50 mM KCl, 2.0 mM $MgCl_2$, 0.001% gelatin, 100 μM dNTPs, 0.5 U of *Taq*DNA polymerase, 0.2 μM each primer, and 4 μl of template DNA. Samples were amplified in a PTC-100 (version 7.0; MJ Research, Inc., Waltham, MA) thermal cycler beginning with denaturation at 94°C for 90 s and 25 cycles of 94°C for 30s, 60°C for 30 s and 72°C for 45 s. PCR products were separated in 2% agarose gels and compared with a 100-bp ladder.

Validation of Diagnostic Primers. All four primer sets were used with specimens from diverse geographic origins (Table 2), testing their reliability and specificity. This included larvae from colonies, larvae from the field, and adults from various locations. In addition, a blind test was performed using three specimens of each species taken from colonies (codling moth, oriental fruit moth, and lesser appleworm) or from fruit (cherry fruitworm collected from blueberries in Michigan) and verified to species based on larval morphology by Enrique Vega, taxonomist from the Mexican Quarantine Diagnostic Laboratory.

Results and Discussion

DNA Extraction and Amplifications. Both QIAGEN and Chelex extraction methods worked well with specimens preserved by various means. DNA was amplified successfully from extractions of fresh, frozen, or fixed (70% ethanol) larval specimens and adults. Using one or two legs, DNA from both pinned museum specimens and from adults removed from sticky pheromone-baited traps was successfully amplified. The universal primer set (525-bp amplicon) worked with 2-yr-old specimens in sticky traps and 8-yr-old museum specimens. The diagnostic primers, which amplify a much smaller fragment (112–116-bp amplicon), were successful with the DNA extracted from the leg of a 58-yr-old specimen, whereas the universal primer set was not. The inability to amplify long fragments from old specimens has been documented (Dean and Ballard 2001), and it has been explained as the breakage and modification of the DNA through time (Lindahl 1993). However, the extraction methods used also may influence the efficacy of amplification of DNA extracted from older specimens. Junqueira et al. (2002) compared DNAzol and Chelex with museum specimens and had a superior recovery of DNA with the former, with the added advantage of longer stability of the resultant DNA sample over time. In our hands, QIAGEN and Chelex protocols were similar for initial amplifications but the Chelex-extracted DNA degraded more rapidly over time.

Sequence Analysis and Primer Design. The universal COI primers C1-J-1718 and C1-N-2191 yielded a single PCR product of ≈525 bp and T_m between 81.8 and 83.4°C, from which a 470-bp core region was sequenced for two codling moth, two Oriental fruit moth, six lesser appleworm, and three cherry fruitworm specimens. Origins of these 13 specimens and their GenBank accession numbers are presented in Table 2. These sequences were supplemented with the codling moth and oriental fruit moth sequences from GenBank from insects collected from the Italian and Austrian Tyrol (R. Dallinger, personal communication). Combining these sequences with ours (Table 2) ensured that DNA sequences from three or more geographically disparate sources of each species were considered during primer design. After alignment and comparison of three to 13 sequences for each species, two regions of COI with interspecific differences in sequence but little or no intraspecific differences were observed, characteristics desirable for designing diagnostic primers (Sperling and Hickey 1995).

Eight primers, two for each species, were designed within regions 2039–2067 (forward) and 2136–2154 (reverse) of COI, where 2–9-bp differences and 1–3-bp differences were observed among species, respectively. Exact location and interspecific comparisons of sequence differences are shown in Table 3. Only geographic populations of lesser appleworm showed intraspecific heterogeneity at the forward diagnostic region and throughout the 470-bp sequence studied, supporting the contention that lesser appleworm is either highly polymorphic or represents multiple species (Chapman and Lienk 1971). In addition, a pair of conserved primers that amplify any of the four *Cydia* or *Grapholita* species was designed to be used for controls (CG-primers): CG-C1-J-1841, 5′-GAAAATGGAGCAGGAACAGG-3′; and CG-C1-N-2099, 5′-ATGATGTATTAAGATTTCGATCTG-3′. These amplified a 304-bp fragment (T_m ≈81.9–82.7°C)

Table 3. Sequence comparison of diagnostic regions and species-specific primers (underlined) used for diagnostic PCR amplification, followed by intra- and interspecific comparison of diagnostic primers with known DNA sequences of each species

Designation[a]	Sequence 5′-3′	No. base pair differences			
		CM	OFM	LAW	CFW
Forward primers (position 2039–2067)[b]					
CM-C1-J-2063	CAGCTCTTTTACTTCTTTTATCATTACCA	0	6	4–6	2
OFM-C1.J-2062	CAGCTCTTTTATTACTACTTTCACTACCA	6	0	6–8	4
LAW-C1-J-2062	CAGCTCTCCTACTTTTACTATCACTACCT	6	6	0–2	8
CFW-C1-J-2067	CAGCTCTTTTATTACTTTTATCATTACCC	3	4	7–9	0
Reverse primers (position 2154–2136)[b]		CM	OFM	LAW	CFW
CM-C1-N-2136	AATAGGATCACCTCCACCA	0	3	3	2
OFM-C1-N-2136	AATAGGATCTCCTCCTCCT	3	0	2	2
LAW-C1-N-2136	AATAGGATCTCCACCACCT	3	2	0	1
CFW-C1-N-2136	AATAGGATCTCCACCACCA	2	3	1	0

[a] Primers are named according to the convention of Simon et al. (1994); species common name initials were added to distinguish primers at identical position. CFW, cherry fruitworm; CM, codling moth; LAW, lesser appleworm; OFM, oriental fruit moth.
[b] *D. yacuba* 5′ position based on sequence of Clary and Wolstenholme (1985).

with the four *Cydia* and *Grapholita* species included in this analysis.

BLAST searches for "short, nearly exact matches" of the eight diagnostic primers showed 100% match for codling moth and oriental fruit moth primers with self COI sequences, and no similarity with any other tortricid (not shown). GenBank contained comparable sequences of COI for 47 tortricid species at the time of this search, including two species within Grapholitini (codling moth and oriental fruit moth) and 12 leafroller species within Archipini known to attack apple and pears around the world.

PCR Amplification with Diagnostic Primers. A unique 112–116-bp fragment was produced when the DNA of a given species was subjected to PCR with their matching diagnostic primers (Figs. 1 and 2). The three nonmatching primer sets either failed to amplify or amplified only a small amount of product by cycle 30 of the PCR. Reactions with matching primers reached the C_T at least 12 cycles earlier than those with nonmatching primers (Fig. 1). No bands were evident with nonmatching primers when products were visualized in agarose gels after 25 cycles of conventional PCR (Fig. 2), but they can be seen as light bands in some gels if PCR is continued for 30 or more cycles (not shown). Both approaches provide unambiguous and reproducible species diagnosis. qPCR with matching primers gave a product that when melted in the RapidCycler, showed a T_m between 80.3 and 81.5°C that corresponded to the ≈115-bp band observed in agarose gels (Fig. 2).

Most DNA-based diagnostics for quarantine applications in arthropods have used restriction enzyme digestion of PCR products generated by amplification with universal primers (PCR-restriction fragment-length polymorphism) (Armstrong et al. 1997, 2003; Lee and Lee 1997; Salazar et al. 2002). PCR with species-specific primers allows diagnosis in a shorter time by avoiding the 2-h or more incubation time required for DNA digestion. Moreover, qPCR, as presented here, provides results in 4 h from the time the sample is received, with 75% of the time associated with sample preparation before PCR. This contrasts with 8–12 h required for conventional PCR. At present, most diagnostic laboratories are equipped for conventional PCR, but the need for quick results for quarantine decision making, the ability to observe qualitative and quantitative differences in the ampli-

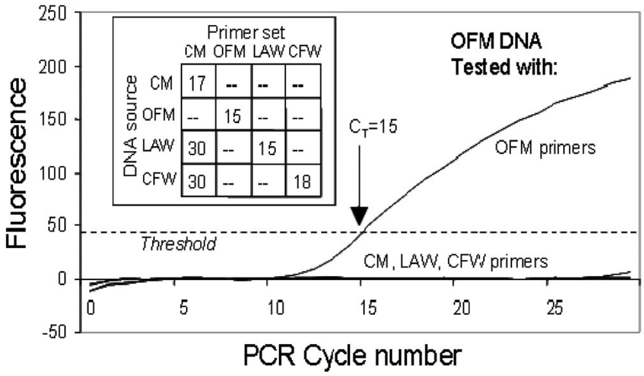

Fig. 1. Example of qPCR output: oriental fruit moth (OFM) DNA used in PCR with codling moth (CM), Oriental fruit moth, lesser appleworm (LAW), and cherry fruitworm (CFW) primer sets. Only OFM DNA with OFM primers shows a characteristic sigmoid curve or a monotonic increase of fluorescence with PCR cycle number. Inset, table of C_T for each combination of DNA and primer set. The symbol –signifies that the fluorescence threshold was not crossed within 30 cycles.

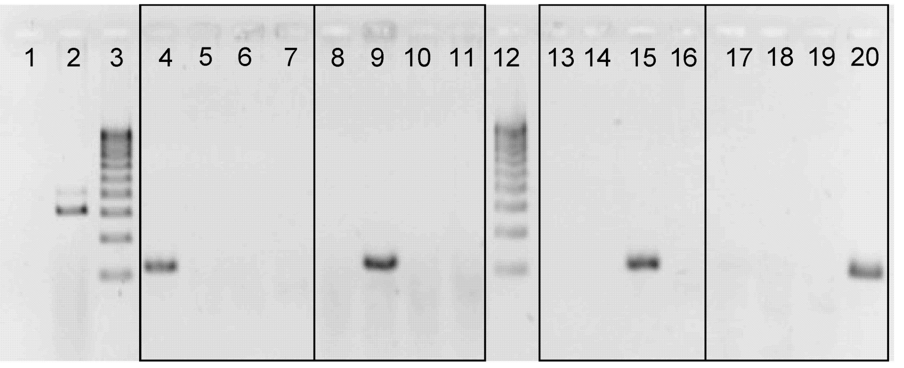

Fig. 2. Conventional PCR products separated in 2% agarose gel and stained with ethidium bromide. Lanes in the gel from left to right represent 1) negative control (no DNA with CG-primers, CG-C1-J-1841 and CG-C1-N-2099); 2) positive control (OFM DNA with CG-primers); 3 and 12) 100-bp ladder; codling moth (CM) DNA with 4) CM primers, 5) oriental fruit moth (OFM) primers, 6) lesser appleworm (LAW) primers, and 7) cherry fruitworm (CFW) primers; OFM DNA with 8) CM primers, 9) OFM primers, 10) LAW primers, and 11) CFW primers; LAW DNA with 13) CM primers, 14) OFM primers, 15) LAW primers, and 16) CFW primers; CFW DNA with 17) CM primers, 18) OFM primers, 19) LAW primers, and 20) CFW primers.

fication reaction, and the cost savings in reduced technical time suggest that qPCR is a useful investment.

Validation of Diagnostic Primers. The one-side blind tests using three specimens of each species (identified and sorted by Enrique Vega) resulted in 100% correct categorization of the species using the diagnostic protocol. Similarly, 25 codling moth, 23 oriental fruit moth, 17 lesser appleworm, and nine cherry fruitworm (Table 2), representing three or more disparate geographic origins, were all identified to species correctly in qPCR assays by using the four primer sets. No exceptions were observed.

This demonstrated that the variation in sequences associated with geographic populations, even when it occurs in the diagnostic region, as discovered in lesser appleworm, did not affect the species specificity and utility of the primer sets. Species also could be diagnosed using specimens of various ages and conditions of preservations, such as with DNA of varied quality and quantity. DNA from a single leg of a 58-yr-old pinned museum specimen or a 2-mm section of a second instar preserved in 70% ethanol amplified successfully and diagnostically. The latter is especially important because it mimics the actual conditions of material shipped for identification from border checkpoints and provides the convenience of keeping the anterior and posterior thirds of the larvae as vouchers for either morphological validation or further DNA analysis.

Caveats and the qPCR Advantage. A protocol for identifying unknown specimens consists of amplifying the DNA by qPCR (or PCR) in 10 reactions: a negative control (no template DNA), a positive "Grapholitini-DNA control" in which DNA of the unknown *Cydia/Grapholita* specimen is amplified with CG-primers as a measure of DNA quality, and four duplicate reactions containing DNA of the unknown by using the four species-specific primer sets. The amplification reaction with species-specific primers, which crosses the C_T 10 or more cycles ahead of the other reactions, represents a species diagnosis. However, this is both conditional on and substantiated by the observed melting temperatures of the products. The melting temperature of the amplified product(s) should correspond to the expected range for the target DNA (80–82°C) and lower melt temperatures characteristic of primer-dimers (73–76°C) should be rejected. Primer-dimer amplifications were occasionally observed, but they crossed the threshold late ($C_T > 24$) and displayed low melting temperatures. Each laboratory must elaborate on this protocol to prevent/detect contamination of primers, buffers, and other reagents in ways that would invalidate comparisons. PCR product from the Grapholitini DNA control cannot be the source of that contamination because the amplified region does not include the four diagnostic reverse primers. Occasional checks of primer quality will require the diagnostic laboratory to have control DNA of each of the target species to ensure that the primer sets are functional. The 525-bp COI PCR product used by us for sequencing and encompassing the 125-bp diagnostic amplicon can be provided to diagnostic laboratories upon written request to T.R.U. Finally, changes in buffer composition or other conditions reported here may affect the C_T and T_M of the products, and users should standardize their results against DNA from known specimens.

High stringency and low number of cycles are important when using conventional PCR with these diagnostic primer sets. Given that the number of differences between diagnostic primers and nontarget sequences is as low as three nucleotides in forward primers and one nucleotide in reverse primers (Table 3), permissive conditions may result in amplification of a nontarget species. In traditional PCR, very light bands on ethidium bromide-stained agarose gels from amplification of a nontarget species contrast with very intense bands from amplification of the target species. One may want to rerun the sample under more stringent conditions (higher annealing temperature) to obtain a more definitive diagnosis. Although both PCR and qPCR can have nonspecific amplifications, interpretation of results is more straightforward with qPCR. With qPCR, even with extremely permissive conditions (favoring nontarget amplifications for some primer/DNA combinations) such as low annealing temperatures and higher number of cycles, the results remain diagnostic. The species-specific reaction passes the C_T value at least 10 cycles sooner than the nontarget reactions.

One shortcoming of our protocol is absence of an internal control; the CG-primers are used to test the quality of unknown DNA, but the PCR is conducted in a separate tube. An anonymous reviewer suggested competitive PCR amplification of specific alleles (PASA) as implemented by Zhu and Clark (1996) (also see Cantafora et al. 1998) to address this issue. In this method, a longer piece of DNA is amplified preferentially as the control DNA, and a shorter piece is additionally amplified only if it is a match with a second forward or reverse primer that is specific to an allele. Competitive PASA is ill suited to qPCR because one or two products cannot be reliably differentiated based on melting curves. Such amplifications should be possible for a gel-based visualization by using our forward diagnostic primers coupled with both the forward and reverse GC primers.

Three assumptions underpin the use of this diagnostic procedure: 1) the unknown specimen was found feeding internally in pome fruit (apples, pears, and quince); 2) it is a Grapholitini tortricid; and 3) it was collected in North America. Other species not fitting these criteria may behave in unpredictable ways with these diagnostic primers. For example, a new species of *Grapholita* closely related to lesser appleworm or cherry fruitworm or a distantly related species of Tortricidae may mistakenly seem to be one of the four target species because its DNA sequence in the two diagnostic regions is similar owing to a close phylogenetic similarity or to similarity arising by chance, respectively. This reflects real life; the tortricid species we found to be most similar in sequence to codling moth at the two diagnostic regions was the obliquebanded leafroller, *Choristoneura rosaceana* (Harris). With 1-bp differences in each the forward and the reverse diagnostic primers compared with codling moth, obliquebanded leafroller DNA produces a product in qPCR by using our recommended conditions at roughly the same C_T as codling moth (data not shown). However, assumptions 1 and 2 are not met: obliquebanded leafroller feeds on, but not in, pome fruits, and it is in a different tribe, Archipini, and can be separated morphologically from the four target species even as small larvae.

Given that the assumptions are met, the diagnostic procedure is robust and useful to nations importing pome fruits from North America. This is the first example of a qPCR protocol applied for molecular diagnostics in moths. It has been validated and approved to be used as a decision making tool by the Quarantine Diagnostic Laboratory of Sanidad Vegetal, Mexico. This tool should help both the U.S. pome fruit industry and importing nations make accurate identifications and appropriate quarantine decisions.

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